4239-56572

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/62, 15/86, 1/21, C07K 19/00 | A2

(11) International Publication Number: **WO 97/24446**

(43) International Publication Date: 10 July 1997 (10.07.97)

(21) International Application Number: PCT/US96/20295

(22) International Filing Date: 20 December 1996 (20.12.96)

(30) Priority Data:
08/580,541 29 December 1995 (29.12.95) US
60/009,411 29 December 1995 (29.12.95) US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GENE DELIVERY VEHICLE-TARGETING LIGANDS

(57) Abstract

Fusion proteins composed of an MHC Class I molecule, MHC Class II molecule, or $\beta 2$ microglobulin, and a targeting ligand are disclosed. Also disclosed are nucleic acid molecules which encode such fusion proteins as well as suitable expression cassettes and host cells. Also provided are methods for targeting a gene delivery vehicle to a selected cell type utilizing gene delivery vehicles which contain on their surfaces one of the above-mentioned fusion proteins.

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GENE DELIVERY VEHICLE-TARGETING LIGANDS

Technical Field

The present invention relates generally to gene delivery vehicles, and more specifically, to methods for targeting gene delivery vehicles.

Background of the Invention

Since the discovery of DNA in the 1940's and continuing through the most recent era of recombinant DNA technology, substantial research has been undertaken in order to realize the possibility that the course of disease may be affected through interaction with the nucleic acids of living organisms. Most recently, a wide variety of methods have been described for altering or affecting genes, including for example, viral vectors derived from retroviruses, adenoviruses, vaccinia viruses, herpes viruses, and adeno-associated viruses (see Jolly, *Cancer Gene Therapy 1*(1):51-64, 1994).

A number of methods have been attempted in order to target viral vectors such as retroviral vectors. For example, Neda et al. (*J. Biol. Chem. 266*(22):14143-14146, 1991) chemically coupled -lactose to viral particles, in order to produce viable viral particles capable of targeting human hepatocytes *in vitro*. Such a method, however, is of limited applicability, and has been only shown to allow the targeting of hepatocytes in tissue cultures.

Others have attempted to link antibodies (Goud et al., *Vir. 163*:251-254, 1988) or antibody fragments (Roux et al., *PNAS 86*:9070-9083, 1989; Etienne-Julan et al., *J. of Gen. Vir. 73*:3251-3255, 1992) with a viral particle, in order to target the viral particle to a specific cell type. Such methods, however, while producing binding of the retrovirus to a specific cell type did not result in the establishment of a proviral state (in Goud et al.) or resulted in only low levels of transduction (Roux et al. and Etienne et al.). Moreover, none of these references described the use of such compositions in order to target cells *in vivo*.

Other attempts have also been made to specifically target a cell type by selecting a vehicle which normally infects that cell type. For example, Shimada et al. (*J. Clin. Invest. 88*:1043-1047, 1991) developed an HIV gene transfer system in order to specifically target CD4+ T cells. One difficulty with such a system, however, is that it produced helper virus (HIV in the above case), which makes such a vector system unsuitable for the treatment of humans.

Other scientists have co-expressed the CD4 protein in-frame with the Avian Leukosis Virus transmembrane protein, or with the transmembrane protein of Murine Leukemia Virus, presumably in an attempt to target HIV infected T cells (Young et al.

Science 250:1421, 1990). While the CD4 protein was expressed by the virus, no evidence was provided which showed that such viral particles were able to transduce target T cells.

In order to more specifically target recombinant gene delivery vehicles, such as retroviruses, particularly those that are delivered *in vivo*, the present invention provides recombinant gene delivery vehicles that are capable of targeting to cells bearing certain specific cell surface molecules or receptors. The present invention provides these, as well as other related advantages.

Summary of the Invention

Briefly stated, the present invention provides compositions and methods for targeting gene delivery vehicles to a selected cell or tissue. Within one aspect of the invention, fusion proteins are provided comprising a MHC Class I molecule, a MHC Class II molecule, or β2 microglobulin and a targeting ligand. Within certain embodiments, the targeting ligand may be an antibody variable region, a hormone such as melanocyte stimulating hormone or erythropoietin.

Within other aspects of the invention, fusion proteins are provided comprising a MHC Class I molecule, a MHC Class II molecule, or β2 microglobulin and one member of a high affinity binding pair. Within one embodiment, the member of a high affinity binding pair is avidin.

Also provided by the present invention nucleic acid molecule (*e.g.*, DNA, RNA, or some combination of the two) which encodes a protein as described above, expression cassettes which are capable of directing the expression of such nucleic acid molecules, and host cells which contain these expression cassettes.

Within other aspects, gene delivery vehicles are provided which have on their surfaces one of the above-described fusion proteins. Representative gene delivery vehicles include recombinant retroviruses and alphaviruses. Within a related aspect, replication defective retroviral vector particles are provided which have a protein comprising heterologous MHC Class II molecule on its surface.

Within other aspects, packaging cell lines are provided comprising a *gag/pol* expression cassette, an *env* expression cassette, and an expression cassette which directs the expression of a sequence encoding one of the above-described fusion proteins. Also provided are vector producing cell lines, which comprise such packaging cell lines and a recombinant retroviral vector.

Within other aspects of the present invention, methods are provided for targeting a gene delivery vehicle to a selected cell type in a warm-blooded animal, comprising the step of administering to a warm-blooded animal one of the above-described gene delivery vehicles. Within a related aspect, such methods comprise the general steps of (a)

administering to a warm-blooded animal a gene delivery vehicle as described above, and (b) administering to the warm-blooded animal a targeting element coupled to a second member of a high affinity binding pair, the coupled targeting element being capable of specifically binding to a selected cell type in said warm-blooded animal, and the second member being capable of specifically binding to the first member, such that the gene delivery vehicle is targeted to the selected cell type.

Within one embodiment, the high affinity binding pair is selected from the group consisting of biotin/avidin, cystatin/papain, val-phosphonate/carboxy-peptidase A and 4CABP/RuBisCo. Within another embodiment, the targeting element may be an antibody variable region or an immune accessory molecule. Within other embodiments, the gene delivery vehicle may be a retroviral vector particle, or liposome or polycation condensed nucleic acids.

Within further embodiments of the invention, the gene delivery vehicle contains a heterologous sequence such as a gene encoding a cytotoxic protein (*e.g.*, ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin and Pseudomonas exotoxin A), an antisense or ribozyme sequence, or an immune accessory molecule (*e.g.*, IL-2, IL-12, IL-15, gamma-interferon, ICAM-1, ICAM-2, β-microglobin, LFA3, and HLA class I and HLA class II molecules). Within other embodiments, the heterologous sequence encodes a gene product that activates a compound with little or no cytotoxicity into a toxic product, such as, for example, HSVTK or VZVTK. Within yet other embodiments, the heterologous sequence may be a replacement gene which encodes a protein such as Factor VIII, ADA, HPRT, CFTCR and the LDL Receptor. In yet another alternative embodiment, the heterologous sequence may encode an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV and HIV.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

Brief Description of the Drawings

Figure 1 is a schematic illustration of pSC6.

Figure 2 is a schematic illustration of pSC6/HLA-A2

Figure 3 provides a representative sequence of an HLA-A2 template.

Figure 4 provides a representative sequence of a gp350/220 peptide.

Figure 5 is a schematic illustration of pCRII/350-A2.

Figure 6 is a diagrammatic illustration of the location of PCR primers selected for amplification of a antibody variable region.

Figure 7 is a schematic illustration of pSC6/HLA-DR alpha.

Figure 8 provides a representative sequence of an HLA DR template.

Figure 9 provides a schematic illustration of pSC6/EPO-β2 M

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

"Gene delivery vehicle" refers to a construct which is capable of delivering, and, within preferred embodiments expressing, one or more gene(s) or sequence(s) of interest in a host cell. Representative examples of such vehicles include viral vectors, nucleic acid expression vectors, naked DNA, and certain eukaryotic cells (*e.g.*, producer cells). Preferably, gene delivery vehicles of the present invention have a molecular weight of greater than about x kilodaltons, wherein x is selected from the group consisting of 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,500, 2,000, 2,500, 3,000, 4,000, and 5,000. Within various aspects of the invention, the gene delivery vehicle has on its surface a fusion protein (discussed below), either expressed on, or included as, an integral part of the exterior of the gene delivery vehicle.

"Vector construct", "retroviral vector", "recombinant vector", and "recombinant retroviral vector" refers to a nucleic acid construct which carries, and within certain embodiments, is capable of directing the expression of a nucleic acid molecule of interest. The retroviral vector must include at least one transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the recombinant retroviral vector may also include a signal which directs polyadenylation, selectable markers such as Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. By way of example, such vectors typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof.

"Recombinant retrovirus", "retroviral gene delivery vehicle" and "retroviral vector particle" as utilized within the present invention refers to a retrovirus which carries at least one gene of interest. The retrovirus may also contain a selectable marker. The

recombinant retrovirus is capable of reverse transcribing its genetic material into DNA and incorporating this genetic material into a host cell's DNA upon infection.

"Nucleic acid expression vector" or "Expression cassette" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. The nucleic acid expression vector must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence. Within certain embodiments of the invention, the nucleic acid expression vectors described herein may be contained within a plasmid construct. In addition to the components of the nucleic acid expression vector, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication).

"Packaging cell" refers to a cell which contains those elements necessary for production of infectious recombinant retrovirus which are lacking in a recombinant retroviral vector. Typically, such packaging cells contain one or more expression cassettes which are capable of expressing proteins which encode *gag*, *pol* and *env* proteins.

"Producer cell" or "Vector producing cell" refers to a cell which contains all elements necessary for production of recombinant retroviral vector particles.

"High Affinity Binding Pair" refers to a set a molecules which is capable of binding one another with a K_D of less than $10^{-y}M$, wherein y is selected from the group consisting of 8, 9, 10, 11, 12, 13, 14 and 15. As utilized herein, the "K_D" refers to the disassociation constant of the reaction $A + B \rightleftharpoons AB$, wherein A and B are members of the high affinity binding pair. (In addition, as should be understood by one of ordinary skill in the art, as the affinity of the two molecules increases, K_D decreases.) Affinity constants may be readily determined by a variety of techniques, including for example by a Scatchard analysis (*see* Scatchard, *Ann. N.Y. Acad. Sci. 51*:660-672, 1949). Representative examples of suitable affinity binding pairs include biotin/avidin, cystatin/papain, phosphonate/ carboxypeptidase A, and 4CABP/RuBisCo.

"Targeting element" refers to a molecule which is capable of specifically binding a selected cell type. As utilized within the context of the present invention, targeting elements are considered to specifically bind a selected cell type when a biological effect of the coupled targeting element may be seen in that cell type, or, when there is greater than a 10 fold difference, and preferably greater than a 25, 50 or 100 fold difference between the binding of the coupled targeting element to target cells and non-target cells. Generally, it is preferable that the targeting element bind to the selected cell type with a K_D of less than $10^{-5}M$, preferably less than $10^{-6}M$, more preferably less than $10^{-7}M$, and most preferably

less than 10^{-8}M (as determined by a Scatchard analysis, *see* Scatchard, *Ann. N.Y. Acad. Sci. 51*:660-672, 1949). In addition it is generally preferred that the targeting element bind to the selected cell type with an affinity of at least 1 log (*i.e.*, 10 times) less than the affinity constant of the high affinity binding pair. (In other words, the K_D value will be at least 1 log or 10 fold greater.) Suitable targeting elements are preferably non-immunogenic, not degraded by proteolysis, and not scavenged by the immune system. Particularly preferred targeting elements (which are conjugated to a member of the high affinity binding pair) should have a half-life (in the absence of a clearing agent) within an animal of between 10 minutes and 1 week. Representative examples of suitable targeting elements are set forth below in more detail.

"Clearing agent" refers to molecules which can bind and/or cross-link circulating coupled targeting elements. Preferably, the clearing agent is non-immunogenic, specific to the coupled targeting element, and large enough to avoid rapid renal clearance. In addition, the clearing agent is preferably not degraded by proteolysis, and not scavenged by the immune system. Particularly preferred clearing agents for use within the present invention include those which bind to the coupled targeting element at a site other than the affinity binding member, and most preferably, which bind in a manner that blocks the binding of the targeting element to its target. Numerous cleaving agents may be utilized within the context of the present invention, including for example those described by Marshall et al. in *Brit. J. Cancer 69*:502-507, 1994.

As noted above, the present invention provides compositions and methods for targeting a gene delivery vehicle to a selected cell type in a warm-blooded animal. Such compositions and methods are based upon the discovery that fusion proteins which are composed of, for example, MHC Class I or Class II molecules, or β2 microglobulin, and a targeting ligand or member of a high affinity binding pair, sort to the surface of a gene delivery vehicle during its construction. Thus, such gene delivery vehicles may be utilized within the methods described herein in order to target the gene delivery vehicle to a specific or selected cell type, based upon the affinity of the targeting ligand (which is present as a fusion protein on the surface of the gene delivery vehicle) for the cell type. Representative examples of suitable fusion proteins and gene delivery vehicles, as well as methods for their preparation and administration, are discussed in more detail below:

A. CONSTRUCTION OF FUSION PROTEINS.

1. *Surface Molecules - MHC Class I Molecules, MHC Class II Molecules, and* β2-*microglobulin*

A wide variety of non-virally encoded cell membrane molecules may be utilized for construction of the fusion protein described herein. In this regard it should be

recognized that although MHC Class I molecules, MHC Class II molecules, and β2 microglobulin are provided as examples, that the present invention is not so limited. In particular, a wide variety of other non-virally encoded virion surface molecules may be utilized, including for example the transferrin receptor, CD43, CD44, CD63, CD71, adhesion molecules such as CD3, CD4, CD11a, CD12, CD13, CD14, CD 15, CD16, CD17, CD18, LF1(CD11a/CD18), CD25, CD54, CD55 (DAF), CD59 (MIRL) or non-human animal equivalents (applies to all the markers).

Briefly, the major histocompatibility complex (MHC) is a region of highly polymorphic genes whose products are expressed on the surface of a variety of cells. The proteins encoded by this complex are termed MHC molecules or MHC antigens and are the principal determinants of graft rejection. There are two major types of MHC gene products: MHC class I and class II molecules, both types of molecules are membrane-bound and present peptide antigens on the cell surface.

Class I molecules contain two separate polypeptide chains; an MHC encoded heavy chain (alpha chain) of approximately 44kD in humans and 47kD in mice, and a non-MHC encoded ß-chain of 12kD. The majority of the heavy chain extends extracellularly, with a short hydrophobic membrane spanning segment and a cytoplasmic carboxy terminal tail of approx. 30 amino-acids. The ß-chain interacts non-covalently with the extracellular portion of the heavy chain and is not directly attached to the cell surface. The class I molecule can be segregated into four separate regions: the peptide-binding region (containing alpha-1 and alpha-2 domains); the Ig-like region; the transmembrane region and the cytoplasmic region. Crystal structure analysis of different MHC class I molecules has shown that the alpha-1 and -2 domains interact to form a platform of an eight stranded ß-pleated sheet supporting two parallel strands of alpha helix. The two alpha helicies form the sides of a cleft whose floor is formed by the ß-sheets. The cleft can bind an 10-20 amino-acid peptide and is presumed to be the site where foreign peptides bind MHC class I for presentation to T-cells.

Comparison of a large number of murine and human Class I molecules has shown that almost all the polymorphic residues are found in the alpha helicies or ß-sheets of the peptide binding groove and are oriented such that the amino-acid side chains point into the groove and are therefore available for binding to the peptide sequence. The Ig-like (alpha-3) region of class I molecules links the peptide binding region to the transmembrane region. This region is highly conserved among all class I molecules examined and shares homology to Ig constant domains. The ß-chain of class I molecules is identical in all human class I molecules. The class I ß-chain is also known as ß2-microglobulin, and like alpha-3 region, contains a disulphide linked loop homologous to an Ig constant domain.

(Guessow et al., *J. Immunol. 139*:3132, 1987) (Robinson et al., *Immunogenetics 20*:655, 1984)

MHC class II molecules are composed of two polypeptide chains termed alpha chain (32-34 kD) and the beta chain (29-32 kD). Both class II chains are polymorphic. The three dimensional structure of Class II molecules has not yet been solved, however, primary sequence analysis reveals structural similarities between class I and class II molecules. Humans possess three class II gene loci encoding DR, DQ and DP molecules. Individual class II genes have previously been isolated and inserted into expression constructs by various investigators, including for example, DQw6A by Nishimura et al., *J. Immun. 145*:353-360, 1990; DRα:DR1ß1, DRα:DR4ß1, DRα:DR5ß1, DRα:DR5ßIII (Drw52), DRα:DR7ß1, DRα:DR4/7ß1V (Drw53), DQ7α:DQw2ß, DQ7α:DQw3ß, and DPw4α:DPw4ß by Klohe et al., *J. Immun.* 141:2158-2164, 1988; and DR2ßa (from DR2Dw2), and DR2ßb (from DR2Dw2) by Wilkinson et al., *J. Exp. Med. 167*:1442-1458, 1988.

2. *High Affinity Binding Pairs*

As noted above, fusion proteins of the present invention may include, in one aspect, one member of a high affinity binding pair. Briefly, a wide variety of high affinity binding pairs may be utilized, including for example, cystatin/papain with an affinity of 10^{-14} M (Bjork and Ylinenjarvi, *Biochemistry 29*:1770-1776, 1990); val-phosponate/carboxypeptidase A with an affinity of 10^{-14} M (Kaplan and Bartlett, *Biochemistry 30*:8165-8170, 1991); 4CABP-RuBisCo with an affinity of 10^{-13} M, (Schloss, *J. Biol. Chem. 263*:4145-4150, 1988); and tobacco hornworm diuretic hormone/tobacco hornworm diuretic hormone receptor, with an affinity of 10^{-11}M (Reagan et al., *Arch. Insect Biochem. Physiol. 23*:135-145, 1993).

A wide variety of other high affinity binding pairs may also be developed, for example, by preparing and selecting antibodies which recognize a selected antigen, and by further screening of such antibodies in order to select those with a high affinity (see generally, U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; see also *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Alternatively, antibodies or antibody fragments may also be produced and selected utilizing recombinant techniques (*see* William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," *Science 246*:1275-1281, December 1989; *see also* L. Sastry et al., "Cloning of the Immunological Repertoire in *Escherichia coli* for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain

Variable Region-Specific cDNA Library," *Proc. Natl. Acad. Sci. USA 86*:5728-5732, August 1989; *see also* Michelle Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," *Strategies in Molecular Biology 3*:1-9, January 1990; these references describe a commercial system available from Stratacyte, La Jolla, California, which enables the production of antibodies through recombinant techniques). The advantage to using the high affinity binding pairs would be generation of targetable vectors from one producer cell with the potential to add any of a cocktail of targeting ligands.

3. *Targeting Ligands*

A wide variety of targeting elements may be utilized within the context of the present invention, in order to specifically direct a gene delivery vehicle to a selected cell type. Generally, targeting elements are proteins or peptides, although other non-proteinaceous molecules may also function as targeting elements. For example, within one embodiment of the invention, antibodies (which include, for example, antibody variable regions) may be utilized in order to target a selected cell type (see generally, Wilchek and Bayer, *Anal. Biochem 171*:1-32, 1988). Representative examples include anti-CD34 antibodies (*e.g.*, 12.8 (Andrews et al., *Blood 67*:842, 1986), and My10 (Civin et al., *J. Immunol. 133*:157, 1984; commercially available from Becton Dickinson under the designation HPCA-2)) which may be utilized to target the anti-CD34 antigen on stem cells, the anti-CD4 antibody which may be utilized to target CD4+ T-cells, anti-CD8 antibodies to target CD8+ cells, the HER2/neu monoclonal antibody 4D5 (Sarup et al., *Growth Regul. 1*:72-82, 1991) to target ovarian and breast cells, the c-erbB-2 monoclonal antibody GFD-OA-p185-1 (Alper et al., *Cell Growth Differ. 1*:591-9, 1990) to target breast cells, the TAG72 monoclonal Ab: CC49 and B72.3 (King et al., *J. Biochem. 281*:317-23, 1992) to target colon and breast cells, and the carcinoembryonic antigen monoclonal antibody ZCE025 (Nap et al., *Canc. Res. 52*:2329-39, 1992) to target colon carcinoma cells.

Other suitable targeting elements include hormones and hormone receptors. Representative examples include follicle stimulating hormone and lutenizing hormone to ovary and testes cells, melanocyte stimulating hormone and epidermal growth factor to epidermal cells, and human growth hormone to mostly bone cells and skeletal muscle cells.

Within other embodiments, immune accessory molecules may be utilized to target specific receptors on various cells. Examples include interferon targeted to macrophages and natural killer cells, interleukins to T-lymphocytes, and erythropoietin and CSF to bone marrow cells.

Within still other embodiments, peptides such as substance P may target neurons as a mediator of pain signals, neuromedin (Conlon, *J. Neurochem. 51*:988, 1988) may be utilized to target the cells of the uterus for contractile activity and proteins corresponding to ligands for known cell surface receptors such as insulin may be utilized to target insulin receptors on cells for glucose regulation.

Within yet other embodiments, other ligands and antibodies may be utilized to target selected cell types, including for example: monoclonal antibody c-SF-25 to target a 125kD antigen on human lung carcinoma (Takahashi et al, *Science 259*:1460, 1993); antibodies to various lung cancer antigens (Souhami, *Thorax 47*:53-56, 1992); antibodies to human ovarian cancer antigen 14C1 (Gallagher et al., *Br. J. Cancer 64*:35-40, 1991); antibodies to $H/Le^y/Le^b$ antigens to target lung carcinoma (Masayuki et al., *N. Eng. J. Med. 327*:14-18, 1992); nerve growth factor to target nerve growth factor receptors on neural tumors (Chao et al., *Science 232*:518, 1986); the Fc receptor to target macrophages (Anderson and Looney, *Immun. Today 1*:264-266, 1987); lectins (Sharon and Lis, *Science 246*:227, 1989); collagen type I to target colon cancer (Pullam and Bodmer, *Nature 356*:529, 1992); Interleukin-1 to target the Interleukin-1 receptor on T cells (Fanslow et al., *Science 248*:739, 1990); acetylated low density lipoproteins ("LDL") to target macrophage scavenger receptors (and atherosclerotic plaques; see Brown et al., *Ann. Rev. Biochem 52*:223-261, 1983), as well as other acetylated molecules which target macrophage scavenger receptors (Paulinski et al., *PNAS 86*:1372-1376, 1989); viral receptors (Haywood, *J. Vir. 68*(1):1-5, 1994); transferrin to target transferrin receptors on tumor cells (Huebers et al., *Physio. Rev. 67*:520, 582, 1987); vasoendothelial growth factor ("vegF") to target cells where increased vascularization occurs; and urokinase plasminogen activator to bind to the urokinase plasminogen activator receptor (UPAR).

Alternatively, ligands may be selected from libraries created utilizing recombinant techniques (Scott and Smith, *Science 249*:386, 1990; Devlin et al., *Science 249*:404, 1990; Houghten et al., *Nature 354*:84 1991; Matthews and Wells, *Science 260*:1113,1993; Nissim et al., *EMBO J. 13*(3):692-698, 1994), or equivalent techniques utilizing organic compound libraries (*e.g.*, Eric Erb et al., *Proc Natl Acad Sci. USA 91*:11422-11426, 1994, K.S. Lam et al., *Nature 354*:82-84, 1991).

Within yet a further embodiment the targeting element or ligand may be a carbohydrate or other non-peptide component, that naturally modifies a particular type of protein sequence. Representative examples include sequences that allow addition of galactose terminal carbohydrates that then would allow targeting to the asyaloglycoprotein receptor on hepatocytes. Within a related embodiment, the targeting element may be added later by chemical or enzymatic methods and this may be facilitated by providing suitable

peptide sequences in the hybrid molecule that favor the chemical or biochemical reaction required to add suitable ligand.

Within preferred aspects of the invention, the targeting ligand may be a chimeric or fusion protein composed of two or more different proteins or non-protein components. Briefly, a protein generally assumes a specific three dimensional structure encoded by its amino acid sequence with a hydrophobic core and a more hydrophilic surface with a defined secondary, tertiary and often quaternary structure. Generally, oligopeptide targeting ligands may be made from portions of a protein sequence that will not assume the full folded structure but may have a portion of the characteristic secondary structure and a characteristic amino acid sequence that defines a particular binding motif. Such binding motif's are generally found in the surface-exposed regions of the protein, such as loops which are found between secondary structure units such as alpha helices or strands of beta sheet. Loops are generally flexible and can be highly variable in length and generally have few constraints on permissible sequence. Other binding regions of proteins may be found in clefts between domains of a single protein, or in regions where different elements of tertiary structure meet.

Generally, in order to add a binding domain to another protein or to change a protein so that it will bind a specified target, a new sequence must be grafted into the existing one so as not to disrupt its structural integrity to such an extent that it cannot fold or be processed and transported properly in the cell. The commonest site of insertion is at the amino or carboxyl end, leaving the rest of the protein intact. Other sites of insertion of new sequence may involve replacement of entire domains of a polypeptide with another domain, such as in the case of the immunoadhesins, where IL-2, for example may replace the variable region of an intact immunoglobulin to make a hybrid molecule. Small peptide sequences may also be inserted internally in proteins particularly at surface-exposed loops or in places where amino acid sequence alignments indicate that a high degree of sequence variability is tolerated or at the amino or carboxy termini.

Representative examples of suitable chimeric or fusion molecules are described in more detail below in the Examples (see Examples 2-5).

B. GENE DELIVERY VEHICLES.

1. *Construction of retroviral gene delivery vehicles*

As noted above, the present invention provides recombinant retroviruses which are constructed to carry or express a selected nucleic acid molecule of interest. Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see RNA Tumor Viruses, Second Edition, Cold Spring

Harbor Laboratory, 1985). Such retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Representative examples of retroviral gene delivery vehicles that may be utilized within the context of the present invention include, for example, those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Ram et al., *Cancer Res. 53*:83-88, 1993; Takamiya et al., *J. Neurosci. Res. 33*:493-503, 1992; Baba et al., *J. Neurosurg. 79*:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO 91/02805). Particularly preferred recombinant retroviruses include those described in WO 91/02805 and WO 95/05789.

Packaging cell lines suitable for use with the above-described vector constructs may be readily prepared (see WO 92/05266), and utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles, given the disclosure provided herein.

2. *Alphavirus delivery vehicles*

The present invention also provides a variety of Alphavirus vectors which may function as gene delivery vehicles. Several different Alphavirus vector systems may be constructed and utilized within the present invention. Representative examples of such systems include those described within WO 95/07994.

3. *Other viral gene delivery vehicles*

In addition to retroviral vectors and Sindbis viral vectors, numerous other viral vectors systems may also be utilized as a gene delivery vehicle. Representative examples of such gene delivery vehicles include viruses such as pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); SV40 (Mulligan et al., *Nature*

277:108-114, 1979); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *N. Eng. J. Med. 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); herpes (Kit, *Adv. Exp. Med. Biol. 215*:219-236, 1989; U.S. Patent No. 5,288,641); HIV (Poznansky, *J. Virol. 65*:532-536, 1991); measles (EP 0 440,219); Semliki Forest Virus, and coronavirus, as well as other viral systems (*e.g.*, EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic(defective), replication competent virus (e.g., Overbaugh et al., *Science 239*:906-910,1988), and nevertheless induce cellular immune responses, including CTL.

4. *Non-viral gene delivery vehicles*

In addition to the above viral-based vectors, numerous non-viral gene delivery vehicles may likewise be utilized within the context of the present invention. Representative examples of such gene delivery vehicles include direct delivery of nucleic acid expression vectors, naked DNA alone (WO 90/11092), polycation condensed DNA linked or unlinked to killed adenovirus (Curiel et al., *Hum. Gene Ther. 3*:147-154, 1992), DNA ligand linked to a ligand with or without one of the high affinity pairs described above (Wu et al., *J. of Biol. Chem 264*:16985-16987, 1989), nucleic acid containing liposomes (*e.g.*, WO 95/24929 and WO 95/12387) and certain eukaryotic cells (*e.g.*, producer cells)

C. HETEROLOGOUS NUCLEIC ACID MOLECULE

A wide variety of nucleic acid molecules may be carried and/or expressed by the expression vectors or recombinant retroviruses of the present invention. Generally, the nucleic acid molecules which are described herein do not occur naturally in the expression vector or recombinant retrovirus that carries it, and provides some desirable benefit, typically an ability to fight a disease, or other pathogenic agent or condition.

Substances which may be encoded by the nucleic acid molecules described herein include proteins (*e.g.*, antibodies including single chain molecules), immunostimulatory molecules (such as antigens) immunosuppressive molecules, blocking agents, palliatives (such as toxins, antisense ribonucleic acids, ribozymes, enzymes, and other material capable of inhibiting a function of a pathogenic agent) cytokines, various polypeptides or peptide hormones, their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. Such polypeptides can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction, immune responses and can be treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or

chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL), levels of Alzheimer associated amyloid protein, bone erosion/calcium deposition, and controlling levels of various metabolites such as steroid hormones, purines, and pyrimidines.

Within one aspect of the present invention, methods are provided for administration of an expression vector or a recombinant retrovirus which directs the expression of a palliative. Representative examples of palliatives that act directly to inhibit the growth of cells include toxins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985).

Within another aspect of the invention, the expression vector or recombinant retrovirus directs the expression of a substance capable of activating an otherwise inactive precursor into an active inhibitor of a pathogenic agent, or a conditional toxic palliative, which are palliatives that are toxic for the cell expressing the pathogenic condition. As should be evident given the disclosure provided herein, a wide variety of inactive precursors may be converted into active inhibitors of a pathogenic agent. For example, expression vectors or recombinant retroviruses which direct the expression of a gene product (*e.g.*, a protein) such as Herpes Simplex Virus Thymidine Kinase (HSVTK) or Varicella Zoster Virus Thymidine Kinase (VZVTK) which assists in metabolizing antiviral nucleoside analogues to their active form are therefore useful in activating nucleoside analogue precursors (*e.g.*, AZT or ddC) into their active form. AZT or ddI therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

In yet another aspect, expression vectors and recombinant retroviruses are provided which have a therapeutic effect by encoding one or more ribozymes (RNA enzymes) (Haseloff and Gerlach, *Nature 334*:585, 1989) which will cleave, and hence inactivate, RNA molecules corresponding to a pathogenic function (*see also*, Foster and Symons, *Cell 48*: 211-220, 1987; Haseloff and Gerlach, *Nature 328*: 596-600, 1988; Ruffner et al., *Biochem 29*: 10,695-10,702, 1990; and PCT Publication No. WO93/23569). In another aspect, expression vectors and recombinant retroviruses are provided comprising a biologically active nucleic acid molecule that is an antisense sequence (an antisense sequence may also be encoded by a nucleic acid sequence and then produced within a host cell via transcription). In preferred embodiments, the antisense sequence is selected from the group consisting of sequences which encode influenza virus, HIV, HSV, HPV, CMV, and HBV. The antisense sequence may also be an antisense RNA complementary to RNA

sequences necessary for pathogenicity. Alternatively, the biologically active nucleic acid molecule may be a sense RNA (or DNA) complementary to RNA sequences necessary for pathogenicity.

Still further aspects of the present invention relate to recombinant retroviruses capable of immunostimulation. Briefly, the ability to recognize and defend against foreign pathogens is essential to the function of the immune system. In particular, the immune system must be capable of distinguishing "self" from "nonself" (*i.e.*, foreign), so that the defensive mechanisms of the host are directed toward invading entities instead of against host tissues. Cytolytic T lymphocytes (CTLs) are typically induced, or stimulated, by the display of a cell surface recognition structure, such as a processed, pathogen-specific peptide, in conjunction with a MHC class I or class II cell surface protein.

In one embodiment, the invention provides methods for stimulating a specific immune response and inhibiting viral spread by using an expression vector or recombinant retroviruses that direct the expression of an antigen or modified form thereof in susceptible target cells, wherein the antigen is capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions.

In another aspect of the invention, the expression vectors or recombinant retroviruses of the present invention may be constructed to express "immunomodulatory factors," many of which are set forth above. Immunomodulatory factors refer to factors that, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the factor. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (*e.g.*, ^{3}H thymidine uptake), and *in vitro* cytotoxic assays (*e.g.*, which measure ^{51}Cr release) (*see*, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*.

Representative examples of such immunomodulatory factors include cytokines or lymhokines, interferons (*e.g.*, γ-IFN), tumor necrosis factors (TNFs) (Jayaraman et al., *J. Immunology 144*:942-951, 1990), CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), ICAM-1 (Altman et al., *Nature 338*:512-514, 1989; Simmons et al., *Nature 331*:624-627, 1988), ICAM-2, LFA-1, LFA-3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), MHC class I molecules, MHC class II molecules, B7.1-.3, β_2-microglobulin (Parnes et al., *PNAS 78*:2253-2257, 1981), chaperones such as calnexin and MHC linked transporter proteins or analogs thereof (Powis et al., *Nature 354*:528-531, 1991).

The present invention also includes expression vectors and recombinant retroviruses which encode immunogenic portions of desired antigens including, for example, viral, bacterial or parasite antigens. Representative examples include hepatitis Band C viral antigens (*e.g.*, WO 93/15207), feline leukemia virus and/or immonudeficiency virus antigens (*e.g.*, WO 94/06921). Still other examples include expression vectors or recombinant retroviruses which direct the expression of a non-tumorigenic, altered genes such as the ras (ras*) gene and the p53 gene (*see* WO 93/10814).

D. CELL CULTURE CONCENTRATION AND PURIFICATION OF RECOMBINANT RETROVIRAL PARTICLES.

As noted above, the present invention provides gene delivery vehicles suitable for administration to humans and other warm-blooded animals. For example, a wide variety of methods may be utilized in order to produce recombinant viruses suitable for administration, including for example, the use of fermenters or bioreactors, roller bottles, cell hotels or cell factories, and hollow fiber culture.

Briefly, for bioreactors or fermenters, cells are preferably grown on microcarriers (*i.e.*, Cytodex 1 or Cytodex 2; Pharmacia, Piscataway, N.J. at concentrations ranging from 3 to 15 g/L microcarrier. For roller bottles, suitable conditions include those described above for bioreactors, with the exception that microcarrier beads are generally not preferred. Representative examples of such methods, as well as other methods such as cell holds or cell factories and hollow linker culture, are described within WO 96/0926.

Subsequent to culturing, a wide variety of methods may be utilized for increasing viral concentration and purity, including for example, precipitation of recombinant retroviruses with ammonium sulfate, polyethylene glycol ("PEG") concentration, concentration by centrifugation (either with or without gradients such as PERCOLL, or "cushions" such as sucrose, use of concentration filters (*e.g.*, Amicon filtration), and 2-phase separations. Representative examples of concentrating and purifying and assigning retroviral particles are described in WO 96/0926.

E. ADMINISTRATION.

As noted above, the present invention provides several methods for the administration gene delivery vehicles. Within one aspect of the present invention, methods are provided for targeting a gene delivery vehicle to a selected cell type in a warm-blooded animal, comprising the step of administering to a warm-blooded animal a gene delivery construct as described above which has a fusion protein containing a targeting ligand on its surface. Within one aspect of the present invention, methods are

provided for targeting a gene delivery vehicle to a selected cell type in a warm-blooded animal, comprising the steps of (a) administering to a warm-blooded animal a gene delivery vehicle which has on its surface a fusion protein having one member of high affinity binding pair, and (b) administering to the animal a gene delivery vehicle coupled to a second member of said high affinity binding pair, the second member being capable of specifically binding to the first high affinity molecule such that the gene delivery vehicle is targeted to the selected cell type. Within one embodiment, such methods further comprise, prior to the step of administering the gene delivery vehicle coupled to a second member of the high affinity binding pair, administering to the animal a clearing agent.

It should be noted that although warm-blooded animals (*e.g.*, humans, macaques, horses, cows, swine, sheep, dogs, cats, chickens, rats and mice) have been exemplified in the methods described above, such methods are also readily applicable to a variety of other animals, including, for example, fish.

Utilizing such methods, gene delivery vehicles of the present invention may be administered to a wide variety of locations including, for example, into sites such as the cerebral spinal fluid, bone marrow, joints, arterial endothelial cells, rectum, buccal/sublingual, vagina, the lymph system, to an organ selected from the group consisting of lung, liver, spleen, skin, blood and brain, or to a site selected from the group consisting of tumors and interstitial spaces. Within other embodiments, the gene delivery vehicles may be administered intraocularly, intranasally, sublinually, orally, topically, intravesically, intrathecally, topically, intravenously, intraperitoneally, intracranially, intramuscularly, or subcutaneously. Other representative routes of administration include gastroscopy, ECRP and colonoscopy, which do not require full operating procedures and hospitalization, but may require the presence of medical personnel.

The above-described methods may be readily utilized for a variety of therapeutic (and prophylactic) treatments. For example, within one embodiment of the invention, the methods described above may be accomplished in order to inhibit or destroy a pathogenic agent in a warm-blooded animal. Such pathogenic agents include not only foreign organisms such as parasites, bacteria, and viruses, but cells which are "foreign" to the host, such as cancer or tumor cells, or other cells which have been "altered". Within a preferred embodiment of the invention, the compositions described above may be utilized in order to directly treat pathogenic agents such as a tumor, for example, by direct injection into several different locations within the body of tumor. Alternatively, arteries which serve a tumor may be identified, and the compositions injected into such an artery, in order to deliver the compositions directly into the tumor. Within another embodiment, a tumor which has a necrotic center may be aspirated, and the compositions injected directly into the now empty center of the tumor. Within yet another embodiment, the above-described

compositions may be directly administered to the surface of the tumor, for example, by application of a topical pharmaceutical composition containing the retrovector construct, or preferably, a recombinant retroviral particle.

Within other aspects of the present invention, methods are provided for generating an immune response against an immunogenic portion of an antigen, in order to prevent or treat a disease, for suppressing graft rejection, for suppressing an immune response, and for suppressing an autoimmune response, utilizing the above-described compositions.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

PREPARATION OF RETROVIRAL VECTOR BACKBONES

Retroviral backbones suitable for use with the present invention may be readily prepared by one of skill in the art. Representative examples of such backbones, such as KT-1 and KT-3, are described in WO 91/02805 and WO 95/05789.

EXAMPLE 2

MHC CLASS I-BASED TARGETING

A. CONSTRUCTION OF THE EXPRESSION BACKBONE, pSC6.

A vector is first created in order to form the backbone for both the *gag/pol* and *env* expression cassettes. Briefly, pBluescript SK- phagemid (Stratagene, San Diego, Calif.; GenBank accession number 52324; referred to as "SK-") is digested with *Spe*I and blunt ended with Klenow. A blunt-ended *Dra* I fragment of SV40 (Fiers et al., *Nature 273*:113-120, 1978) from *Dra* I (bp 2366) to *Dra* I (bp2729) is then inserted into SK-, and a construct isolated in which the SV40 late polyadenylation signal is oriented opposite to the lacZ gene of SK-. This construct is designated SK-SV40A.

A Human Cytomegalovirus Major Immediate Early Promoter ("HCMV-IE"; Boshart et al., *Cell 41*:521-530, 1985) (*Hin*cII, bp 140, to *Eag*I, bp814) is isolated after digestion with *Hin*cII and *Eag*I, and the *Eag*I site blunt ended. The 674 blunt ended fragment is ligated into SK-SV40A. The final construct, designated pSC6 is then isolated

(see Figure 1). This construct contains the HCMV promoter oriented in opposite orientation to the lacZ gene, and upstream from the late polyadenylation signal of SV40.

B. CLONING MHC CLASS I, SPECIFICALLY HUMAN HLA-A2.

The human HLA-A2 gene is isolated as a 4 kb *Hind* III - *Apa*L1 fragment (Koller and Orr, *J. Immun. 134*(4):2727-2733, 1985) and inserted into expression construct pSC6 (Invitrogen) using the *Hind* III site and the *Eco*R V sites in the polylinker of pSC6 (Figure 1). The expression construct contains the human CMV immediate early promoter driving the human HLA-A2 gene. The *Apa*LI site is treated with T4 DNA polymerase to create a blunt end and the fragment is ligated to the pSC6 cut with *Hind* III and *Eco*R V (Figure 2). This construct is designated pSC6/HLA-A2.

C. FUSION OF HUMAN HLA-A2 WITH THE PEPTIDE FROM EBV GP350/220: EDPGFFNVE AT THE MATURE AMINO TERMINUS.

PCR primers are synthesized with the sequences specified in Table I in order to fuse the gp350/220 sequence to the HLA-A2 template at the mature amino terminus, and the PCR reactions are run as indicated in Table II according to the manufacturer's instructions with a GeneAmp PCR kit (Perkin/Elmer) (Figures 3 and 4). The PCR product containing the gp350/220 peptide sequence at the amino terminus of HLA-A2 is cloned into the vector pCRII according to the manufacturer's instructions using the TA cloning kit (Invitrogen, San Diego, CA) (Figure 5). The sequence of the PCR product is verified by standard DNA sequencing methods. The fragment is removed from pCRII by digestion with the *Eco*47 III and *Bsp*EI sites and cloned into the pSC6/HLA-A2 vector (Figure 2) at the *Eco*47 III and *Bsp*EI sites to make the fusion vector, pSC6/350-A2.

Table I

Sequences of Primers for gp350/220 Peptide Fusion to HLA-A2 Amino Terminus

Primer	Sequence	Seq. ID No.
A	GACATTGAGACAGAGCGCTT	1
B	GGGGCTCTGGCCCTGGAAGATCCAGGATTTTTCAAT	2
C	CGCCCAGGTCTGGGTATCAACATTGAAAAATCCTGG	3
D	GAGGGTCCGGAGTATTGGGA	4

Table II

Insertion of EBV gp350/220 Peptide with HLA-A2 Amino Terminus by Single Overlap PCR

Template	Primer 1	Primer 2	Product
HLA-A2	A	C	AC
HLA-A2	B	D	BC
AC and BD	A	D	Final Insert

D. CONSTRUCTION OF A PACKAGING CELL LINE CONTAINING MOLONEY ECOTROPIC ENVELOPE.

The ecotropic envelope expression vector, pSC6/eco is created by inserting the *Xba*I-*Nhe*I fragment of MoMLV (bp 5766 through bp 7845 of MoMLV) into pSC6 expression vector (Example 2A). Briefly the *Xba*I-*Nhe*I envelope fragment is isolated from pMLV-K (Miller et al., *J. Vir. 49*:214-222,1988) on an agarose gel. The fragment is then blunt-ended with T4 polymerase using standard methods, ligated into pSC6, digested at the *Eco*RV and *Sma*I sites. The product in the correct orientation has a HCMV promoter followed by the complete ecotropic envelope coding sequence and an SV40 polyadenylation signal.

The ecotropic packaging cell line 293E is made by cotransfecting 293 *gag-pol* ("293 2-3"; WO 91/02805; Burns, *et al*, PNAS *90*: 8033-8037, 1993) with the pSC6/eco vector and pSV2gpt (Mulligan and Berg, *Science 209*:1422, 1980). After selection for gpt positive cells in the presence of selection medium, individual resistant colonies are isolated by dilution cloning and analyzed for expression by western blot analysis using goat polyclonal antibody for gp70 (NCI/BCB Repository Serum Number 79S000842, 1667 Davis St, Camden, NJ 08104).

E. TRANSFECTION OF PSC6/350-A2 INTO 293E, OR 293 2-3, TO MAKE A PACKAGING CELL LINE.

The *gag/pol* and MoMLV ecotropic envelope cell line, 293E, or the gag/pol cell line 293 2-3, is co-transfected by $CaPO_4$ precipitation (Wigler, M. et al., *Cell 11*:223, 1977) with 10 μg of pSC6/350-A2 and 1 ug of the phleomycin resistance vector, pUT507 (Mulsant et al., *Somat. Cell Mol. Genet. 14*:243-52, 1988). After selection for transfected cells in the presence of medium containing 10 μg/ml phleomycin, individual drug resistant cell colonies are expanded and analyzed for expression by western blot using monoclonal antibodies for HLA-A2.

F. GENERATION OF A PRODUCER CELL LINE USING A THYMIDINE KINASE VECTOR.

1. *Retroviral vector constructs*

The gene coding for thymidine kinase (ATP:thymidine 5' phosphotransferase, EC 2.7.1.21) of *Herpes simplex* virus type 1 is obtained from the plasmid p322TK (Enquist et al., *Gene* 7:335-342, 1979; Wagner et al., *P.N.A.S.* 78:1441-1445, 1981). The promoter and the polyadenylation signal are removed and the gene is further modified to generate a 5' *Xho* I site and a 3' *Cla* I site. This modified gene fragment is then cloned into the *Xho* I and *Cla* I sites of the KT-3 backbone to generate the BH-1 vector. In the BH-1 (N2/tk) vector, the ATG methionine initiator codon for MoMLV *gag* expression has been mutated to ATT to inhibit *gag* expression (Chada et al., *J. Vir.* 67(6):3409-3417, 1993). The MoMLV long terminal repeat (LTR) controls the expression of the HSV-tk gene and an internal SV40 promoter drives the expression of the neomycin resistance marker.

2. *Vector production*

The retroviral packaging cell line, DA (Irwin et al., *J. Vir.* 68(8):5036-5044, 1994; Laube et al., *Hum. Gene. Ther.* 5:853-862, 1994), is engineered to express the murine leukemia virus *gag/pol* and amphotropic env sequences in the canine sarcoma cell line, D17. The DA/N2/tk producer cell lines are generated by transduction of vesicular stomatitis virus G pseudotyped vector (Burns et al., *P.N.A.S.* 90:8033-8037, 1993) into the DA packaging cell line followed by selection in G418 (800 μg/ml). Individual clones are isolated by dilution cloning and tested for $G418^R$ titer. The best producer clones (based on titer) DA/BH-1 #9A and #18A are selected for subsequent use. These DA/N2/tk producer cells are grown to confluence, and supernatants containing the replication-defective N2/tk vectors are collected, filtered (pore size 0.45 um) and stored at -80°C. The vector titers are determined by serial dilution and CFU assay (Irwin et al., 1994) on HT-1080 indicator cells selected in medium containing G418 (800 ug/ml). Titers are generally between 10^5 and 10^6 cfu/ml. The DA/β-gal producer cell line has been described previously (Irwin et al., 1994).

In a similar manner, the packaging cell line 293E/350-A2 (Example 2E) is used to generate a producer cell line by transducing with vesicular stomatitis virus G pseudotyped vector (Burns et al., *P.N.A.S.* 90:8033-8037, 1993) into the 293E/350-A2 packaging cell line followed by selection in G418 (400 μg/ml). Individual clones are isolated by dilution cloning and tested for $G418^R$ titer. The best producer clones (based on titer) are selected for subsequent use. These producer cells are grown to confluence, and supernatants containing the replication-defective N2/tk vectors are collected, filtered (pore size 0.45

um) and stored at -80°C. The vector titers are determined by serial dilution (Irwin et al., 1994) on HT-1080 indicator cells selected in medium containing G418 (800 ug/ml).

Vectors may be introduced into other cell lines as described above, and clones similarly selected.

G. TARGETING RAJI CELLS WITH THYMIDINE KINASE VECTOR.

This experiment uses the B lymphoblastoid cell line, Raji (ATCC CCL 56), which expresses the complement C3d receptor, CD21, as a target for transduction by the HLA fusion protein/ thymidine kinase vector and U937 (ATCC CRL 1593) a monocyte-like cell line (CD21 minus) as a control.

Two six well dishes are set up with 1 x 10^6 of either Raji cells or U937 cells in five wells. The wells are exposed to (1) thymidine kinase vector produced in an amphotropic packaging cell line, DA, (2) thymidine kinase vector produced in the HLA-A2 fusion vector packaging cell line, (3) thymidine kinase vector produced in the packaging cell line 293E, or 293 2-3, (4) irrelevant vector, DA/ßgal, (5) no vector. Half of the cells are stained for thymidine kinase production and subjected to FACS analysis for production of thymidine kinase. The other half are subjected to treatment with 3, 6 or 9 µg/ml gancyclovir for 3-5 days and counted to determine percent of viable cells remaining. Alternatively, gancyclovir efficacy can be determined using relative levels of ^3H thymidine uptake (Bradley, L.M., in Selected Methods in Cellular Immunology, pp. 156-161, (B. Mishell and S. Shiigi, eds.), Freeman & Co., N.Y. (1980).

EXAMPLE 3

MHC CLASS II-BASED TARGETING

A. CLONING AND EXPRESSION OF MHC CLASS II DR, DQ AND DP GENES.

1. *Expression of MHC class II proteins in retroviral producer cells*

Many different vectors exist for expression of MHC class II gene products. For example, Wilkinson, D et al., *J. Exp. Med. 167*: 1442-1458, 1988 describe the construction of vectors expressing DR2ßa and DR2ßb from the DR2Dw2 cell line PGF.

Expression vectors encoding the different Class II genes (both alpha and beta chains may be combined in the same construct, or transfected individually) are transfected into DA/ß gal cells using the $CaPO_4$ procedure (described above) and transfected cells isolated by FACS sorting for cell surface expression of the appropriate Class II molecule (see below for details). Initial experiments determine whether all three class II alleles can

be expressed in canine DA cells. Pools of selected cells are expanded and ß-gal (V) are isolated from confluent cultures.

2. *Detection of HLA class II molecules bound to virions*

HLA class II specific monoclonal antibodies are obtained from Pharmingen (San Diego, CA) and include H143 (anti-DP), Tu169 (anti-DQ1, DQ2), Tu36 (anti-DR) or Caltag IIb3 (anti Dqw1; Caltag, San Francisco, CA), 7.3.19.1 (anti-DRw52) or HL-37 (anti DQ1, DQ3). The antibodies are bound to magnetic beads (Dynal Inc., New Hyde Park, New York) according to the manufacturers instructions and used to purify ß-gal(V) from the different producer cell lines. Vector bound to the beads is quantitated by ELISA using an anti-p30 detection antibody.

Alternatively, affinity columns may be prepared by binding the anti-class II monoclonal antibodies to sepharose using the amino-link kit according to the manufacturers instructions (Pierce, Rockford Illinois). Vector supernatants are passed over the columns and bound vector eluted using 1M NaCl and titered for ß-gal activity on HT 1080 cells. This assay can detect as little as one functional ß-gal virion. Vector from the DA/ß-gal-DR cells is passed over the DR; DP and DQ columns and specificity of binding evaluated. Similar experiments are performed for vectors from control DA/ß gal cells as well as from DA/ß gal-DP and DA/ß gal-DQ cells.

To assess preferential sorting of class II molecules to virion surfaces, DP, DQ and DR are transfected into DA/ß gal cells and the resulting vector are tested as above for binding activity to class II specific columns.

B. CLONING V REGION OF IGG, ANTI-TRANSFERRIN RECEPTOR MONOCLONAL ANTIBODY, 454A12.

The variable regions of Mab 454A12 (U.S. Patent No. 4,938,948) are cloned from the hybridoma cell line (ATCC HB10804). The heavy and light variable regions are obtained from the mRNA by PCR (Larrick et al., *BioTechniques* 7:934-838 (1989)) using mixed oligonucleotide primers that correspond to the leader sequences and the constant region sequences. The products of the PCR are cloned into pCRII using the TA Cloning kit (Invitrogen) and sequenced by the Sanger dideoxynucleotide method. Three clones are sequenced in full to verify the DNA sequence of each V region. The sequences are given in Tables III and IV.

C. CONSTRUCTION OF ANTI-TRANSFERRIN RECEPTOR ANTIBODY, 454A12, SINGLE CHAIN FV FRAGMENT.

The heavy and light chain variable regions are assembled to form a single chain Fv fragment with a synthetic linker sequence consisting of three repeats of Gly-Gly-Gly-Gly-Ser between them. The primers, and templates for the assembly of the 454A12 sFv are summarized in Tables III through V. The products of the two PCR reactions are used as templates in overlap PCR (Horton, R. M. et al., *Biotechniques 8*:528-535 (1990)) as shown in Table VI. The overlap PCR product is then cloned into the vector pCRII via the TA cloning kit (Invitrogen) as previously described and corresponds to the single chain Fv fragment of approximately 750 bp in length. Several clones are subjected to Sanger dideoxynucleotide sequencing to obtain a clone of the correct predicted DNA sequence.

D. CONSTRUCTION OF THE EXPRESSION VECTOR FOR THE MHC-ANTIBODY FUSION PROTEIN WITH THE ANTI-TRANSFERRIN RECEPTOR, 454A12, SINGLE CHAIN FV FRAGMENT.

The DR gene is inserted into the pSC6 vector at the *Eco*RV and *Eco*RI sites in the polylinker as a three piece ligation of the HLA DR gene cut from *Sca* I to *Pst* I (4 Kb fragment), and *Pst* I to *Eco*RI (1700 bp fragment). The *Sca* I creates a blunt end which ligates to the *Eco*R V site of the polylinker. The exon containing the signal peptide and mature amino terminus is subjected to double overlap PCR from *Nde* I to *Bgl* II to add the sFv (described above section) at the amino terminus using primers described in Table VII and PCR reactions described in Table VIII. The final product is cloned into pCRII for sequence verification. The amino terminal *Nde* I to *Bgl* II fragment is cloned into the pSC6/HLA-DR to make pSC6/DR-aTfR.

Table III

Sequence of 454A12 Heavy chain V Region Template

Seq. ID No. 9

GAGGTCCAGCTGCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGGCT
TCTGGCTACACCTTCAGATCCTACTATATACACTGGGTGAGGCAGAGGCCTGGACAGGGACTTGAGTGGATT
GGTTGGATTTATCCTGGAGATGGTAATACTCTGTCCAATGAGAAGTTCAAGGGCAAGACCACTCTGACTGCA
GACAAATCCTCCAACACAGCCTACATGTTCCTCAGCAGCCTGGCCTCTGAGGACTCTGCGATCTATTTCTGT
GCAAGAGATACTACGACTACCGGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCT

Table IV

Sequence of 454A12 Light chain V Region Template

Seq. ID No. 10

GAGCTCGTGATGACACAGTCTCCATCCTCCCTGACTGTGACAGCAGGAGATAAGGTCACTATGAGCTGCAAG
TCCAGTCAGAGTCTTTTAAACAATGGAAATCAAAAGAACTTCTTGACCTGGTACCAGCAGAAACCAGGGCAG
CCTCCTAAACTTTTGATCTACTGGGCATCCACTAGGGATTCTGGGGTCCCTGATCGCTTCACAGGCAGTGGA
TCTGGAACAGATTTCACTCTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCATATTATTACTGTCAGAAT
GATTATAGTTATCCTCTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA

Table V

Sequences of Primers for PCR Overlap of 454A12 sFv Construction

Primer	Sequence	Seq. ID No.
UF	CTCGAGGTCCAGCTGCAGCAG	5
DB	CGACCCACCACCGCCCGAGCCACCGCCACCAGCAGAGACAGTGAC	6
UT	GGTGGTGGGTCGGGTGGCGGCGGATCTGAGCTCGTGATGACAC	7
DF	TGGTGCAGCATCAGCCCGTTTCAG	8

Table VI

Assembly of 454A12 sFv by Overlap PCR

Template	Primer 1	Primer 2	Product
454A12 V Heavy	UF	DB	VL plus link
454A12 V Light	UT	DF	VH plus link
VL and VH	UF	DF	454A12 sFv

Table VII

Sequences of Primers for Anti-Transferrin Receptor Antibody 454A12 sFv Fusion to HLA-DR Amino Terminus

Primer	Sequence	Seq. ID No.
A	TCATTGCACAGACATATGAT	11
B	CAGGAATCATGGGCTGAGGTCCAGCTGCAG	12
C	CTGCAGCTGGACCTCAGCCCATGATTCCTG	13
D	AAGCTGGAGCTGAAAAAGCTGGAGCTGAAA	14
E	TTTCAGCTCCAGCTTATCAAAGGTAGGTGC	15
F	ATATAAGATCTGCTGTACCA	16

Table VIII

Insertion of 454A12 sFv Fusion at the HLA-DR Amino Terminus by Double Overlap PCR

Template	Primer 1	Primer 2	Product
HLA-A2	A	C	AC
454A12 sFv	B	E	BE
AC and BE	A	E	AE
HLA-A2	D	F	DF
AE and DF	A	F	final product

E. TRANSFECTION OF pSC6/DR-aTfR INTO 293E, OR 293 2-3, TO MAKE A PACKAGING CELL LINE.

The *gag/pol* and MoMLV ecotropic envelope cell line, 293E, or cell line 293 2-3, is co-transfected by $CaPO_4$ precipitation (Wigler, M. et al., *Cell 11*:223, 1977) with 10 µg of pSC6/DR-aTfR and 1 ug of the phleomycin resistance vector, pUT507 (Mulsant et al., *Somat. Cell Mol. Genet. 14*:243-52, 1988). After selection for transfected cells in the presence of medium containing 10 µg/ml phleomycin, individual drug resistant cell colonies are expanded and analyzed for expression by western blot using HLA class II DR-specific monoclonal antibody, Tu36, obtained from Pharmingen (San Diego, CA).

F. ß-GALACTOSIDASE VECTOR

The bacterial lacZ gene encoding β-galactosidase ("βgal"; Price, *PNAS 84*:156, 1987) is cloned into the N2 backbone as described (Irwin et al., 1994) to generate the N2 βgal provector plasmid. N2 ß gal provector is used to generate a stable producer cell clone termed DA/ß-gal. Purified vector supernatant has been generated from this cell line and concentrated to titers of greater than $1X10^8$ cfu/ml.

G. TRANSDUCTION OF HLA DR-ANTIBODY FUSION PROTEIN PCL WITH ß GALACTOSIDASE VECTOR.

The packaging cell line 293E/DR-aTfR, or, 293 2-3/DR-aTfR, is used to generate a producer cell line by transducing with vesicular stomatitis virus G pseudotyped vector (Burns et al., *P.N.A.S. 90*:8033-8037, 1993) into the 293E/DR-aTfR packaging cell line, followed by selection in G418 (400 µg/ml). Individual clones are isolated by dilution cloning and tested for $G418^R$ titer. The best producer clones (based on titer) are selected for subsequent use. These producer cells are grown to confluence, and supernatants containing the replication-defective N2-ßGal vectors are collected, filtered (pore size 0.45 um) and stored at -80°C. The vector titers are determined by serial dilution (Irwin et al., 1994) on HT-1080 indicator cells selected in medium containing G418 (800 ug/ml).

H. TARGETING TO TRANSFERRIN RECEPTOR ON CELLS.

This experiment uses the cell line Molt4 (ATCC CRL1582) which expresses the transferrin receptor as a target for transduction by the HLA DR-antiTfR/ßGal vector. Briefly, two six well dishes are set up with 1×10^5 of MOLT4 cells in four wells. The wells are exposed to (1) ßgal vector produced in the amphotropic packaging cell line, DA (Example 3E), (2) ßgal vector produced in the HLA-A2 fusion vector packaging cell line, (3) ßgal vector produced in the packaging cell line 293E (Example 2D), (4) no vector. Half of the cells are stained for ßgal production. The other half are subjected to treatment with 1000 µg/ml geneticin for 10 days and live cells counted (Irwin, et al., 1994, *supra*). Targeting is demonstrated by the presence of either blue cells or G418 resistant colonies, as described in (2) above.

EXAMPLE 4

β2 MICROGLOBULIN (β2M)-BASED TARGETING

A. CLONING OF HUMAN ERYTHROPOIETIN (EPO) AND CONSTRUCTION OF AN EXPRESSION VECTOR WITH EPO FUSED TO HUMAN β2M.

The basic strategy employed for the generation of the EPO-b2m fusion protein is to use PCR with the appropriate primers and templates to produce EPO and b2m fragments which can then be ligated with the appropriately digested eukaryotic expression vector pSC6.

In particular, a three way ligation is accomplished by utilizing a *Cla*I-*Aat*I EPO fragment, an *Aat*I-*Eco*RI b2m fragment and a *Cla*I-*Eco*RI- digested pSC6 expression vector.

The plasmid EPOenv-D5923 (Kasahara et al., *Science 266*:1373, 1994) is utilized as the source of the human EPO gene. This clone contains cDNA which encodes the complete mature EPO protein of 166 amino acids in length and which can be excised intact from the vector using the restriction enzymes *Bst*E II and *BamH* I. The *Bst*E II site is treated with T7 polymerase to create a blunt end and the fragment is then ligated with *Eco*RV and *BamH*I digested plasmid SK+ (Stratagene, La Jolla, CA). This plasmid construct is termed SK+/EPO and the presence of a *Cla*I site 5' to the *Eco*RV site in the multicloning site of SK+ allows the EPO gene to be removed from the SK+/EPO plasmid as a *Cla*I-*Aat*I fragment. The *Aat*I site is a unique restriction site located near the end of the 5th exon of the EPO gene and is located before the termination codon.

Next, the human b2m gene fragment is engineered to contain an *Aat*I restriction site at its 5' end and an *Eco*RI restriction site at its 3' end. The plasmid p714 (Parker and Wiley, *Gene 83*:117, 1989) containing cDNA encoding human b2m serves as the PCR template for a set of primers which will amplify a gene fragment containing the b2m gene (minus its signal peptide) as well as all the necessary sequences to fuse the 3' end of the EPO gene (minus its termination codon) in frame with the b2m gene. Specifically, the primer set has been designed as follows: Primer 1: 5' GGGAGGCCTG CAGGACAGGG GACAGACAGC GTACTCCAAA GATTCAGGTT 3' (Seq. ID No. 17) and contains the *Aat*I restriction site, the last several codons of the EPO gene and the first several codons of the b2m gene. Primer 2 is the antisense sequence containing the *Eco*RI restriction site and the last 9 codons of the b2m gene, (5' GGGAATTCTA CCTGGCGCTG TTACATGTCT CGGTC 3' (Seq. ID No. 18). The PCR reaction is run according to manufacturer's instructions with a Gene Amp PCR kit (Perkin/Elmer) and the resulting amplified gene product is cloned into the vector pCRII according to the manufacturer's instructions using the TA cloning kit (Invitrogen, San Diego, CA). The sequence of the amplified PCR

product is verified using standard DNA sequencing methods. The fragment is then removed from pCRII by digestion first with *Aat*I and then by partial digestion with *Eco*RI (there is an additional *Eco*RI site 209 bp 5' from the terminal *Eco*RI which is located just before the termination codon of the b2m gene). Pilot digestions are set up to determine the optimal conditions for digesting the terminal *Eco*RI site while leaving the other *Eco*RI site undisturbed. Once the correct *Aat*I-*Eco*RI b2m fragment has been isolated, it is ligated simultaneously with the *Cla*I-*Aat*I EPO gene fragment as well as with *Cla*I-*Eco*RI digested pSC6 vector DNA. The resulting fusion construct pSC6/EPO-b2 is sequenced to verify that the EPO-b2m gene fusion is correct.

B. TRANSFECTION OF PSC6/EPO-B2M INTO 293E OR 293 2-3 TO MAKE A PACKAGING CELL LINE.

The *gag/pol* and MoMLV ecotropic envelope cell line, 293E (Example 2D) or 293 2-3 is co-transfected by $CaPO_4$ precipitation (Wigler et al., *Cell 11*:223, 1977) with 10 mg of pSC6/EPO-b2m plasmid DNA and 1 ug of the phleomycin resistance vector, pUT507 (Mulsant et al., *Somat. Cell Mol. Genet. 14*:243-52, 1988). After selection for transfected cells in the presence of phleomycin, individual drug resistant cell colonies are expanded and analyzed for expression by western blotting and fluorescent staining using monoclonal antibodies for EPO and for human b2m. Alternatively, cells expressing high levels of EPO-b2m may be selected using a Fluorescence Activated Cell Sorter (FACS). Specifically, for EPO detection, polyclonal antiserum to EPO (AB-286-NA; R & D Systems, Minneapolis, MN) are used and for b2m detection, mAb BBM-1, an antibody which binds an epitope present in both native and denatured b2m (Parham, et al., *J. Biol. Chem. 258*: 6179-6186, 1983) are used.

C. GENERATION OF PRODUCER CELL LINE USING B-GALACTOSIDASE VECTOR.

The packaging cell line 293E/EPO-b2m, or 293 2-3/EPO-b2m, is used to generate a producer cell line by transducing the packaging cell line with vesicular stomatitis virus G pseudotyped vector (Burns et al., *P.N.A.S. 90*:8033-8037, 1993), followed by selection in G418 (400 µg/ml). Individual clones are isolated by dilution cloning and tested for $G418^R$ titer. The best producer clones (based on titer) are selected for subsequent use. These producer cells are grown to confluence, and supernatants containing the replication-defective N2-ßGal vectors are collected, filtered (pore size 0.45 um) and stored at -80°C. The vector titers are determined by serial dilution (Irwin et al., 1994) on HT-1080 indicator cells selected in medium containing G418 (800 ug/ml).

D. TARGETING TO EPO RECEPTORS.

This experiment uses a cell line in which NIH 3T3 cells are stably transfected with a complementary DNA (cDNA) encoding the EPO receptor (cDNA source is A. D'Andrea et al., *Cell 57*: 277, 1989). Standard transfection and selection methodologies are used to isolate NIH 3T3 cells stably expressing the EPO receptor (NIH 3T3-EPOR) and FACS analysis is used to confirm the expression of the EPO receptor on these cells. Two six well dishes are set up with 1 x 10^5 of the NIH 3T3-EPOR cells or NIH 3T3 cells (does not contain EPO receptor) in four wells. The wells are then exposed to (1) bgal vector produced in an amphotropic packaging cell line, DA, (2) βgal vector produced in the EPO-b2m fusion vector packaging cell line, (3) βgal vector produced in the packaging cell line 293E, (4) no vector. Half of the cells are stained for bgal production. The other half are subjected to treatment with 600 ug/ml geneticin for 10 days and live cells counted (Irwin, et al., 1994).

EXAMPLE 5

(B2M)-BASED TARGETING BY SUBUNIT EXCHANGE

A. CLONING OF EPO-B2M FUSION PROTEIN INTO A PROKARYOTIC EXPRESSION VECTOR.

The recombinant EPO-b2m fusion protein may be expressed using the prokaryotic expression vector pSE280 (Invitrogen, San Diego, CA). The EPO-b2m gene is excised from pSC6/EPO-b2m as a *Cla*I-*Eco*RI fragment by performing the *Cla*I digestion first, treating with klenow to generate a blunt end, followed by an *Eco*RI-partial digestion. This fragment is then ligated with *Nco*I-*Eco*RI digested pSE280 vector DNA, (*Nco*I end is also blunted prior to setting up the ligation). This recombinant plasmid is termed pSE280/EPO-b2m.

B. EXPRESSION OF EPO-B2M FUSION PROTEIN FOR SCALE UP AND PURIFICATION.

Briefly, the pSE280 plasmid containing the EPO-b2m fusion protein (pSE280/EPO-b2m) is transformed into *E. coli* strain XA90 and positive protein-producing clones are then sequenced to verify their DNA sequence. Subsequently, positive clones are grown to the appropriate density and induced with isopropyl b-D-thiogalactopyranoside (IPTG;1mM). When cells reach a density at OD_{650} of 1.8-2.0, the cultures are harvested (1 liter aliquots) by centrifugation and subsequently lysed using a solution of 10mM Tris-HCl, pH 8 (20 ml/1 liter of pelleted cells), containing lysozyme at 100 ug/ml, the protease inhibitor PMSF at 50 ug/ml, DNase at 20 ug/ml, RNase at 20 ug/ml, and 1 mM EDTA. Cells are incubated in this mixture for 20 min. at RT and then

sonicated before another round of centrifugation at 10,000 x g for 20 min. The resulting pellet containing the recombinant protein is washed with 20 ml of 10mM Tris-HCl, pH 8 and then resuspended in solution of 100mM Tris-HCl, pH 8 and 8M urea (10ml total). Following resuspension, the lysate is centrifuged at 150,000 x g for 1hr at 4°C. The recombinant fusion protein in urea is then dialyzed against 10 mM Tris-HCl, pH7, and purified on Q Sepharose in 10mM Tris-HCl, pH7, with a linear gradient from 0-100 mM NaCl. Fractions are collected and concentrated by ultrafiltration using Centricon filters (Amicon).

C. EXCHANGE OF EPO-B2M FUSION PROTEIN WITH NATIVE B2M ON THE VIRAL SURFACE.

In order to determine b2 exchange, purified EPO-b2m fusion proteins are iodinated using the chloramine-T method (Hunter, W.M., (1973), in Handbook of Experimental Immunology, ed., Weir, D.M. (Blackwell, Oxford), Vol. 1, pp. 17.1-17.36) or using the Bolton-Hunter reagent (Bolton, and Hunter *Biochem. J. 133*: 529-539, 1973). The former method results in specific activities of 10^3 cpm/ng whereas the latter method produces 2-5 x 10^4 cpm/ng. Briefly, 5 x 10^5 HT1080 cells in 1 ml of RPMI medium containing gelatin at 1 mg/ml and puromycin at 50 ug/ml are incubated with 20 ug of labeled b2m for 4 hr at 37°C. The cells are washed 3 times with Hanks buffered saline (HBSS) and the final cell pellet is lysed using lysis buffer routinely used for immunoprecipitation. Immunoprecipitation reactions use the BBM-1 monoclonal antibody which is directed against an epitope present in both native and denatured b2m. After immunoprecipitation of the labeled cells, the radioactive label is detected indicating that the labeled b2m had been exchanged with the native b2m associated with these cells.

Purified EPO-b2m fusion protein is exchanged with the b2m present on the vector surface as described above. Following the exchange reaction, this "exchanged" vector is tested for targeting to cells expressing the EPO receptor, (as described in example 4D). Amicon filtration (300k molecular weight cutoff) is used to purify the vector from contaminating substances.

D. TARGETING TO EPO RECEPTORS.

Two six well dishes are set up with 1 x 10^5 of the NIH 3T3-EPOR cells or NIH 3T3 cells (does not contain EPO receptor) in four wells. The wells are then exposed to (1) βgal vector produced in an amphotropic packaging cell line, DA, (2) βgal vector produced as in (1) but following the EPO-b2m fusion protein exchange, (3) βgal vector produced in the packaging cell line 293E, (4) no vector. Half of the cells are stained for bgal

production. The other half are subjected to treatment with 600 ug/ml geneticin for 10 days and live cells counted (Irwin et al., 1994).

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Chiron Viagene, Inc.

(ii) TITLE OF INVENTION: Gene Delivery Vehicle-Targeting Ligands

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:
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(D) STATE: California
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(F) ZIP: 94608

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 1190-100

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(A) TELEPHONE: (510) 923-3520
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(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GACATTGAGA CAGAGCGCTT 20

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGGCTCTGG CCCTGGAAGA TCCAGGATTT TTCAAT 36

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCCCAGGTC TGGGTATCAA CATTGAAAAA TCCTGG 36

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGGGTCCGG AGTATTGGGA 20

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCGAGGTCC AGCTGCAGCA G 21

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGACCCACCA CCGCCCGAGC CACCGCCACC AGCAGAGACA GTGAC 45

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGTGGTGGGT CGGGTGGCGG CGGATCTGAG CTCGTGATGA CAC 43

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGGTGCAGCA TCAGCCCGTT TCAG 24

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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TCCTGCAAGG CTTCTGGCTA CACCTTCAGA TCCTACTATA TACACTGGGT GAGGCAGAGG 120

CCTGGACAGG GACTTGAGTG GATTGGTTGG ATTTATCCTG GAGATGGTAA TACTCTGTCC 180

AATGAGAAGT TCAAGGGCAA GACCACTCTG ACTGCAGACA AATCCTCCAA CACAGCCTAC 240

ATGTTCCTCA GCAGCCTGGC CTCTGAGGAC TCTGCGATCT ATTTCTGTGC AAGAGATACT 300

ACGACTACCG GGTTTGCTTA CTGGGGCCAA GGGACTCTGG TCACTGTCTC TGCT 354

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 339 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAGCTCGTGA TGACACAGTC TCCATCCTCC CTGACTGTGA CAGCAGGAGA TAAGGTCACT 60

ATGAGCTGCA AGTCCAGTCA GAGTCTTTTA AACAATGGAA ATCAAAAGAA CTTCTTGACC 120

TGGTACCAGC AGAAACCAGG GCAGCCTCCT AAACTTTTGA TCTACTGGGC ATCCACTAGG 180

GATTCTGGGG TCCCTGATCG CTTCACAGGC AGTGGATCTG GAACAGATTT CACTCTCACC 240

ATCAGCAGTG TGCAGGCTGA AGACCTGGCA TATTATTACT GTCAGAATGA TTATAGTTAT 300

CCTCTCACGT TCGGTGCTGG GACCAAGCTG GAGCTGAAA 339

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCATTGCACA GACATATGAT 20

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGGAATCAT GGGCTGAGGT CCAGCTGCAG 30

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTGCAGCTGG ACCTCAGCCC ATGATTCCTG 30

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGCTGGAGC TGAAAAAGCT GGAGCTGAAA 30

(2) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTTCAGCTCC AGCTTATCAA AGGTAGGTGC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATATAAGATC TGCTGTACCA 20

(2) INFORMATION FOR SEQ ID NO:17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGGAGGCCTG CAGGACAGGG GACAGACAGC GTACTCCAAA GATTCAGGTT 50

(2) INFORMATION FOR SEQ ID NO:18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGAATTCTA CCTGGCGCTG TTACATGTCT CGGTC 35

(2) INFORMATION FOR SEQ ID NO:19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 707 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GACATTGAGA CAGAGCGCTT GGCACAGAAG CAGAGGGGTC AGGGCGAAGT CCAGGGCCCC 60

AGGCGTTGGC TCTCAGGGTC TCAGGCCCCG AAGGCGGTGT ATGGATTGGG GAGTCCCAGC 120

CTTGGGGATT CCCCAACTCC GCAGTTTCTT TTCTCCCTCT CCCAACCTAT GTAGGGTCCT 180

TCTTCCTGGA TACTCACGAC GCGGACCCAG TTCTCACTCC CATTGGGTGT CGGGTTTCCA 240

GAGAAGCCAA TCAGTGTCGT CGCGGTCGCG GTTCTAAAGT CCGCACGCAC CCACCGGGAC 300

TCAGATTCTC CCCAGACGCC GAGGATGGCC GTCATGGCGC CCCGAACCCT CGTCCTGCTA 360

CTCTCGGGGG CTCTGGCCCT GACCCAGACC TGGGCGGGTG AGTGCGGGGT CGGGAGGGAA 420

ACGGCCTCTG TGGGGAGAAG CAACGGGCCG CCTGGCGGGG GCGCAGGACC CGGGAAGCCG 480

CGCCGGGAGG AGGGTCGGGC GGGTCTCAGC CACTCCTCGT CCCCAGGCTC TCACTCCATG 540

AGGTATTTCT TCACATCCGT GTCCCGGCCC GGCCGCGGGG AGCCCCGCTT CATCGCAGTG 600

GGCTACGTGG ACGACACGCA GTTCGTGCGG TTCGACAGCG ACGCCGCGAG CCAGAGGATG 660

GAGCCGCGGG CGCCGTGGAT AGAGCAGGAG GGTCCGGAGT ATTGGGA 707

(2) INFORMATION FOR SEQ ID NO:20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1133 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CATATGATCT GTATTTAGCT CTCACTTTAG GTGTTTCCAT TGATTCTATT CTCACTAATG 60

TGCTTCAGGT ATATCCCTGT CTAGAAGTCA GATTGGGGTT AAAGAGTCTG TCCGTGATTG 120

ACTAACAGTC TTAAATACTT GATTTGTTGT TGTTGTTGTC CTGTTTGTTT AAGAACTTTA 180

CTTCTTTATC CAATGAACGG AGTATCTTGT GTCCTGGACC CTTTGCAAGA ACCCTTCCCC 240

TAGCAACAGA TGCGTCATCT CAAAATATTT TTCTGATTGG CCAAAGAGTA ATTGATTTGC 300

ATTTTAATGG TCAGACTCTA TTACACCCCA CATTCTCTTT TCTTTTATTC TTGTCTGTTC 360

TGCCTCACTC CCGAGCTCTA CTGACTCCCA AAAGAGCGCC CAAGAAGAAA ATGGCCATAA 420

GTGGAGTCCC TGTGCTAGGA TTTTTCATCA TAGCTGTGCT GATGAGCGCT CAGGAATCAT 480

GGGCTATCAA AGGTAGGTGC TGAGGGAATG AAATCTGGGA CGATAGACTA CGAAGCATTG 540

GAGAAAAGAC CTATGGACAT TTGGAAGATA ATGTGTGGAG TGAAAGAATA GTGTGACAGG 600

TATTATGTGG TCTCGACAGA AAGTATAACA AATTGTGGTT TGGTGGAGTT CTTCCCTCAC 660

CACAAACTGA AGTAAGTCAA ATTTGGTTTA GAGGGTCAAA ACTGAGTTGT GTATTGATGA 720

ATAGCACGGT CCTGCTACAA GCCAAACTGG GGGTGGGGGT GGGGGTGGGG GAGGAAGAAT 780

ATTTTCTGGC AAGCATTAAC AAGTTATATT TCTGGGCTTT AATTATTCTT TCTGGAAAAT 840

TAGTAAAATT AAAAACTAAA AACCACACAT AGTTTTGTTA GAATTAAATG AAAAAAAAAG 900

TTATTAGCCC TGTTCTTATC TGAATACATG ATACAGTAGT TATTTTTTGG AGTGTAAATC 960

CTGTCGGTAT ATATTGAGCA CATATATTGT GTTGAAGATT ACTAGAAGGA AAAGTCATCA 1020

AAAAGCAACA ATTTACCCCA GGAAAAGGGG AGGGAAGGCA TGCTGATATG AGTTGCCTCA 1080

TGGGACAGTG ATAGCCATTC CCTGCCTTCC CATCTCCATG GTACAGCAGA TCT 1133

Claims

We Claim:

1. A fusion protein, comprising a non-virally encoded cell membrane molecule and a targeting ligand.

2. A fusion protein, comprising a MHC Class I molecule and a targeting ligand.

3. A fusion protein, comprising a MHC Class II molecule and a targeting ligand.

4. A fusion protein, comprising β2 microglobulin and a targeting ligand.

5. The fusion protein according to any one of claims 1 to 3 wherein said targeting ligand is an antibody variable region.

6. A fusion protein, comprising a MHC Class I molecule and one member of a high affinity binding pair.

7. A fusion protein, comprising a MHC Class II molecule and one member of a high affinity binding pair.

8. A fusion protein, comprising β2 microglobulin and one member of a high affinity binding pair.

9. The fusion protein according to any one of claims 7 to 8 wherein said member of a high affinity binding pair is avidin.

10. A nucleic acid molecule which encodes a protein according to any one of claims 1 to 9.

11. An expression cassette which directs the expression of a nucleic acid molecule according to claim 10.

12. A host cell containing an expression cassette according to claim 11.

13. A gene delivery vehicle which has on its surface a fusion protein according to any one of claims 1 to 5.

14. A gene delivery vehicle which has on its surface a fusion protein according to any one of claims 6 to 9.

15. A replication defective retroviral vector particle which has a protein comprising heterologous MHC Class II molecule on its surface.

16. A packaging cell line, comprising a *gag/pol* expression cassette and an expression cassette according to claim 11.

17. A vector producing cell line, comprising a packaging cell line according to claim 16 and a recombinant retroviral vector.

18. A method for targeting a gene delivery vehicle to a selected cell type in a warm-blooded animal, comprising administering to a warm-blooded animal a gene delivery vehicle according to claim 13.

19. A method for targeting a gene delivery vehicle to a selected cell type in a warm-blooded animal, comprising:

(a) administering to a warm-blooded animal a gene delivery vehicle according to claim 14; and

(b) administering to said warm-blooded animal a targeting element coupled to a second member of a high affinity binding pair, said coupled targeting element being capable of specifically binding to a selected cell type in said warm-blooded animal, and said second member being capable of specifically binding to said first member, such that said gene delivery vehicle is targeted to said selected cell type.

FIGURE 1

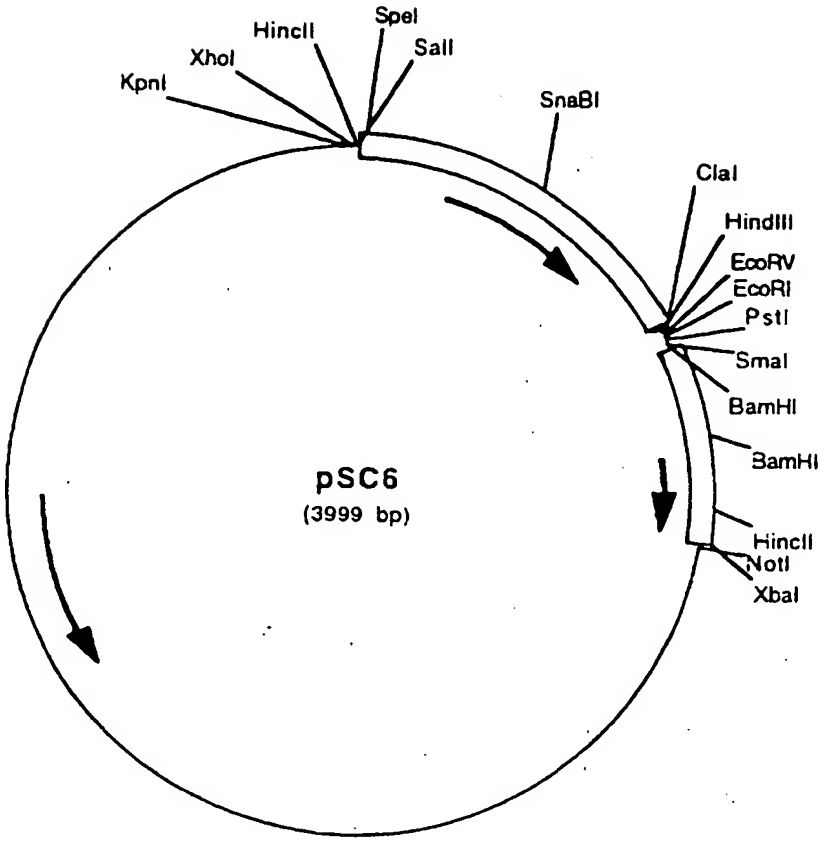

HincII
SpeI
XhoI
SalI
KpnI
SnaBI
ClaI
HindIII
EcoRV
EcoRI
PstI
SmaI
BamHI
BamHI
pSC6
(3999 bp)
HincII
NotI
XbaI

FIGURE 2

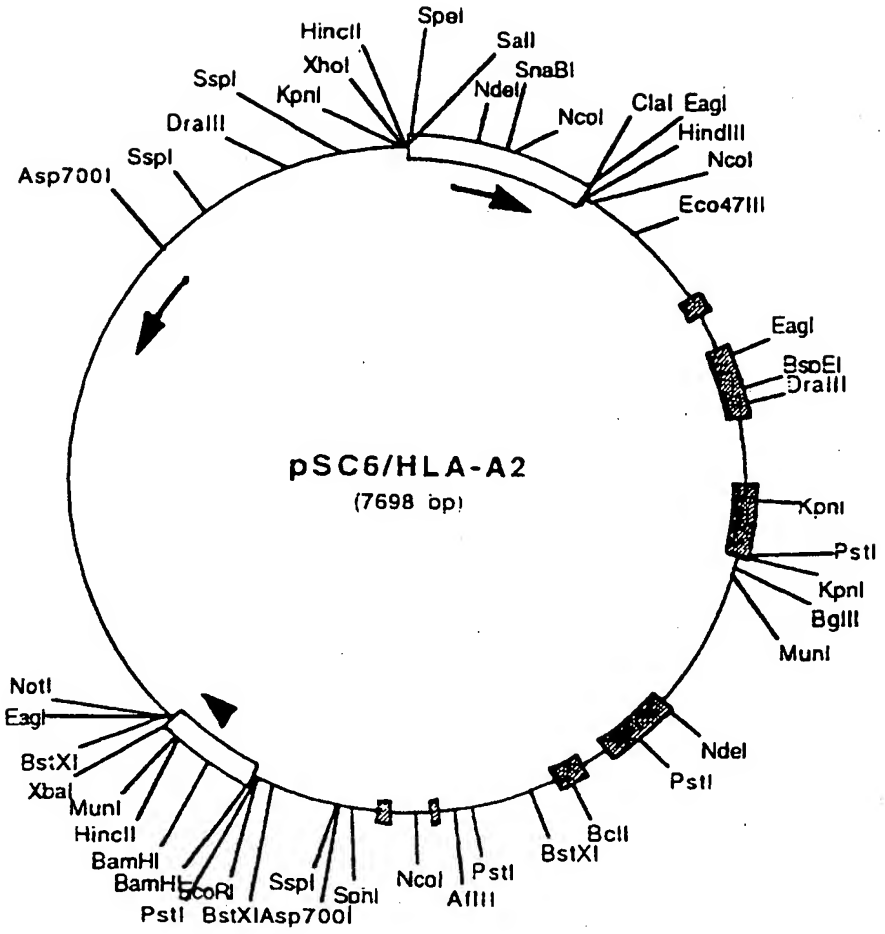

pSC6/HLA-A2
(7698 bp)
HincII
SpeI
SalI
XhoI
SnaBI
SspI
KpnI
NdeI
ClaI EagI
DraIII
NcoI
HindIII
SspI
NcoI
Asp700I
Eco47III
EagI
BspEI
DraIII
KpnI
PstI
KpnI
BglII
MunI
NotI
EagI
NdeI
PstI
BstXI
XbaI
BclI
MunI
BstXI
HincII
BamHI
NcoI
PstI
BamHI
EcoRI
SspI
AflII
SphI
PstI
BstXIAsp700I

FIGURE 3

Sequence of HLA-A2 Template

1 GACATTGAGA CAGAGCGCTT GGCACAGAAG CAGAGGGGTC AGGGCGAAGT
51 CCAGGGCCCC AGGCGTTGGC TCTCAGGGTC TCAGGCCCCG AAGGCGGTGT
101 ATGGATTGGG GAGTCCCAGC CTTGGGGATT CCCCAACTCC GCAGTTTCTT
151 TTCTCCCTCT CCCAACCTAT GTAGGGTCCT TCTTCCTGGA TACTCACGAC
201 GCGGACCCAG TTCTCACTCC CATTGGGTGT CGGGTTTCCA GAGAAGCCAA
251 TCAGTGTCGT CGCGGTCGCG GTTCTAAAGT CCGCACGCAC CCACCGGGAC
301 TCAGATTCTC CCCAGACGCC GAGGATGGCC GTCATGGCGC CCCGAACCCT
351 CGTCCTGCTA CTCTCGGGGG CTCTGGCCCT GACCCAGACC TGGGCGGGTG
401 AGTGCGGGGT CGGGAGGGAA ACGGCCTCTG TGGGGAGAAG CAACGGGCCG
451 CCTGGCGGGG GCGCAGGACC CGGGAAGCCG CGCCGGGAGG AGGGTCGGGC
501 GGGTCTCAGC CACTCCTCGT CCCCAGGCTC TCACTCCATG AGGTATTTCT
551 TCACATCCGT GTCCCGGCCC GGCCGCGGGG AGCCCCGCTT CATCGCAGTG
601 GGCTACGTGG ACGACACGCA GTTCGTGCGG TTCGACAGCG ACGCCGCGAG
651 CCAGAGGATG GAGCCGCGGG CGCCGTGGAT AGAGCAGGAG GGTCCGGAGT
701 ATTGGGA

FIGURE 4

Sequence of gp350/220 Peptide

1 GAAGATCCAG GATTTTTCAA TGTTGAT

FIGURE 5

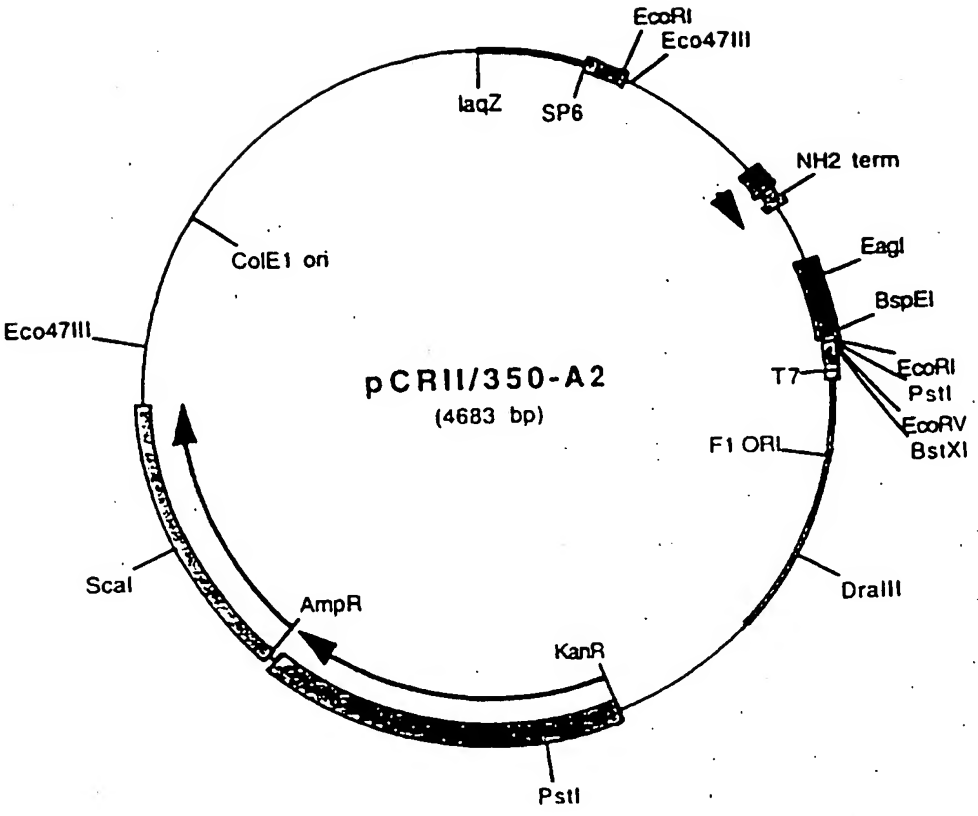

EcoRI
Eco47III
lacZ
SP6
NH2 term
EagI
BspEI
EcoRI
PstI
EcoRV
BstXI
T7
F1 ORI
DraIII
KanR
PstI
AmpR
ScaI
Eco47III
ColE1 ori
pCRII/350-A2
(4683 bp)

FIGURE 6

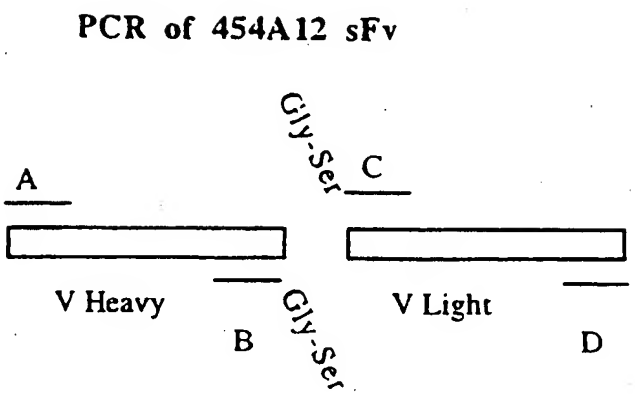


FIGURE 7

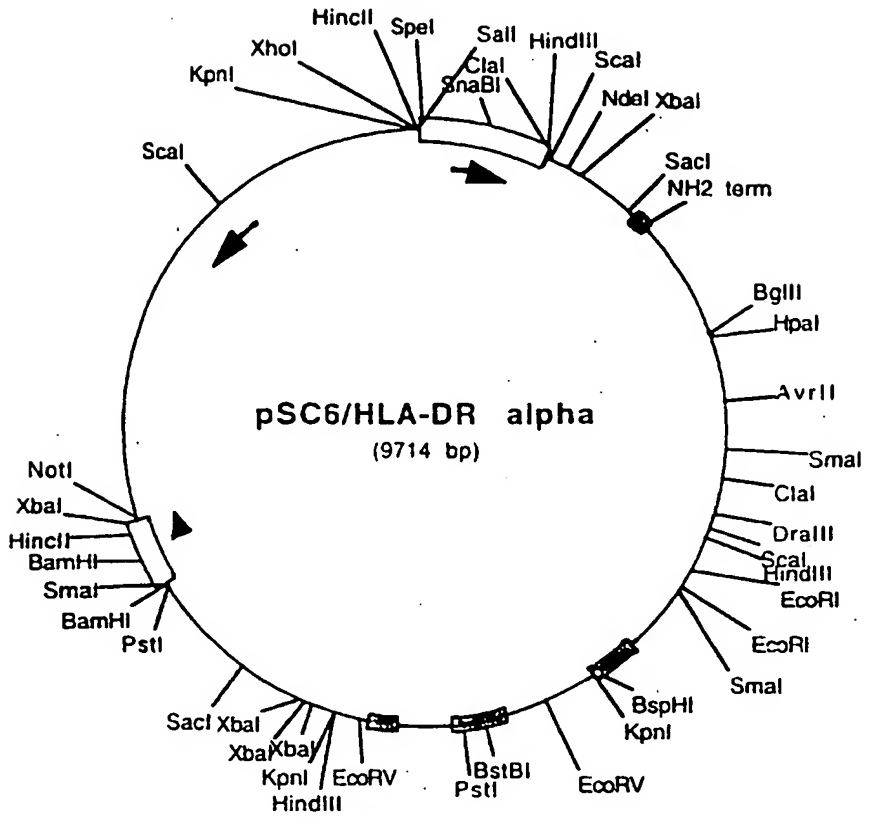
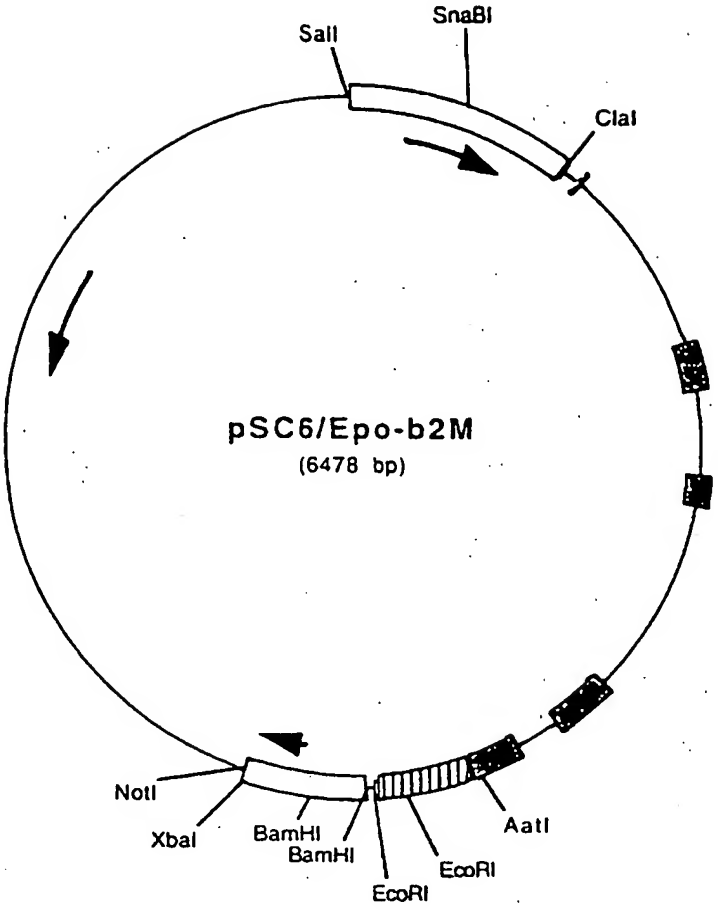

pSC6/HLA-DR alpha
(9714 bp)
HincII
SpeI
SalI
HindIII
ScaI
XhoI
ClaI
KpnI
SnaBI
NdeI
XbaI
SacI
NH2 term
ScaI
BglII
HpaI
AvrII
SmaI
ClaI
DraIII
ScaI
HindIII
EcoRI
EcoRI
SmaI
BspHI
KpnI
EcoRV
BstBI
PstI
SacI
XbaI
XbaI
XbaI
KpnI
EcoRV
HindIII
NotI
XbaI
HincII
BamHI
SmaI
BamHI
PstI

FIGURE 8

HLA DR alpha template sequence

1 CATATGATCT GTATTTAGCT CTCACTTTAG GTGTTTCCAT
41 TGATTCTATT CTCACTAATG TGCTTCAGGT ATATCCCTGT
81 CTAGAAGTCA GATTGGGGTT AAAGAGTCTG TCCGTGATTG
121 ACTAACAGTC TTAAATACTT GATTTGTTGT TGTTGTTGTC
161 CTGTTTGTTT AAGAACTTTA CTTCTTTATC CAATGAACGG
201 AGTATCTTGT GTCCTGGACC CTTTGCAAGA ACCCTTCCCC
241 TAGCAACAGA TGCGTCATCT CAAAATATTT TTCTGATTGG
281 CCAAAGAGTA ATTGATTTGC ATTTTAATGG TCAGACTCTA
321 TTACACCCCA CATTCTCTTT TCTTTTATTC TTGTCTGTTC
361 TGCCTCACTC CCGAGCTCTA CTGACTCCCA AAAGAGCGCC
401 CAAGAAGAAA ATGGCCATAA GTGGAGTCCC TGTGCTAGGA
441 TTTTTCATCA TAGCTGTGCT GATGAGCGCT CAGGAATCAT
481 GGGCTATCAA AGGTAGGTGC TGAGGGAATG AAATCTGGGA
521 CGATAGACTA CGAAGCATTG GAGAAAAGAC CTATGGACAT
561 TTGGAAGATA ATGTGTGGAG TGAAAGAATA GTGTGACAGG
601 TATTATGTGG TCTCGACAGA AAGTATAACA AATTGTGGTT
641 TGGTGGAGTT CTTCCCTCAC CACAAACTGA AGTAAGTCAA
681 ATTTGGTTTA GAGGGTCAAA ACTGAGTTGT GTATTGATGA
721 ATAGCACGGT CCTGCTACAA GCCAAACTGG GGGTGGGGGT
761 GGGGGTGGGG GAGGAAGAAT ATTTTCTGGC AAGCATTAAC
801 AAGTTATATT TCTGGGCTTT AATTATTCTT TCTGGAAAAT
841 TAGTAAAATT AAAAACTAAA AACCACACAT AGTTTTGTTA
881 GAATTAAATG AAAAAAAAAG TTATTAGCCC TGTTCTTATC
921 TGAATACATG ATACAGTAGT TATTTTTTGG AGTGTAAATC
961 CTGTCGGTAT ATATTGAGCA CATATATTGT GTTGAAGATT
1001 ACTAGAAGGA AAAGTCATCA AAAAGCAACA ATTTACCCCA
1041 GGAAAAGGGG AGGGAAGGCA TGCTGATATG AGTTGCCTCA
1081 TGGGACAGTG ATAGCCATTC CCTGCCTTCC CATCTCCATG
1121 GTACAGCAGA TCT

FIGURE 9


SnaBI
SalI
ClaI
pSC6/Epo-b2M
(6478 bp)
NotI
XbaI
BamHI
BamHI
EcoRI
EcoRI
AatI